*NEGATIVE COOPERATIVITY IN REGULATORY ENZYMES**

By Alexander Levitzki† and D. E. Koshland, Jr.

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated January 24, 1969

Abstract.—Negative cooperativity has been observed in CTP synthetase, an allosteric enzyme which contains a regulatory site. Thus, the same enzyme exhibits negative cooperativity for GTP (an effector) and glutamine (a substrate) and positive cooperativity for ATP and UTP (both substrates). In the process of the delineation of these phenomena, diagnostic procedures for negative cooperativity were developed. Application of these procedures to other enzymes indicates that negative cooperativity is a characteristic of many of them. These findings add strong support for the sequential model of subunit interactions which postulates that ligand-induced conformational changes are responsible for regulatory and cooperative phenomena in enzymes.

Recently it has been shown that phosphoenolpyruvate carboxylase[1] and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase[2] exhibit negative cooperativity in the binding of substrate. This type of cooperativity had been predicted from the sequential model[3] of subunit interactions and evidence had been presented that ligand-induced conformational changes were responsible for the pattern of these enzymes. Three questions immediately arise. First, is negative cooperativity a rare event that occurs in a few enzymes with unusual structural features or is it an ubiquitous feature of enzymes? Second, how can one recognize such behavior in other enzymes which may be behaving in a similar way? Third, is the phenomenon associated with certain types of enzymes, for example those which lack regulatory sites, or certain types of small molecules, for example substrates? The findings reported here on an enzyme containing regulatory sites and exhibiting positive homotropic effects may throw light on these questions.

CTP synthetase has been isolated and purified by Long and Pardee[4] and has been shown to catalyze the reaction of equation (1).[5–7]

$$\text{UTP} + \text{ATP} + \text{glutamine} \xrightarrow[Mg^{++}]{\text{GTP}} \text{CTP} + P_i + \text{glutamic acid} + \text{ADP}. \quad (1)$$

The enzyme exhibits positive homotropic effects toward ATP and UTP with Hill coefficients of 3.8 and 3.2, respectively.[4] It has recently been shown that the active enzyme is a tetramer of molecular weight 200,000.[7] In the examination of the kinetic properties of this enzyme, negative cooperative effects have been uncovered. The evaluation of these phenomena in relation to the structure of the protein has led to diagnostic procedures which have proved helpful in understanding the nature of negative cooperativity.

Materials and Methods.—The tetralithium salts of UTP, ATP, and GTP were purchased from Schwarz BioResearch.

Glutamine was a product of Calbiochem. All other chemicals used were of analytical grade.

The enzyme CTP synthetase was prepared according to Long and Pardee,[4] and kept in

0.02 M sodium phosphate buffer, pH 7.4, containing 2 mM glutamine and 1 mM EDTA at 4°C. Prior to use the enzyme was dialyzed against 0.02 M imidazole-acetate buffer, pH 7.2, containing 0.05 M β-mercaptoethanol. The activity was measured as described previously,[4] except that imidazole-acetate buffer, 0.02 M, pH 7.2, was used instead of Tris-HCl.[8]

Results.—The initial velocity of CTP synthesis was determined as a function of glutamine concentration in the presence of different fixed GTP concentrations (Fig. 1). It can be seen that at the lowest GTP concentration a pronounced deviation from the Michaelis-Menten hyperbola occurs. Increased concentrations of GTP decrease these deviations until at the highest GTP level (2.5×10^{-4} M) only a minor deviation from the Michaelis-Menten pattern is observed. This can be seen either by comparing the R_s values,[3] i.e., the ratio of the 90–10 per cent saturation velocities, or by plotting the same data on a double-reciprocal plot as shown in Figure 2. In Figure 3 the double-reciprocal plot of the effect on reaction velocity of varying GTP concentrations is presented. GTP was shown by Long and Pardee to be an effector of the reaction,[4] and has been shown to activate the enzyme without itself decomposing.[7] The GTP produces pronounced deviations from straight lines and reveals the same biphasic dependence as glutamine. With GTP the enzyme responds to the effector as if it possessed two dissociation constants, $K_1 = 4 \times 10^{-5}$ M and $K_2 = 1 \times 10^{-4}$ M.

To understand these results it is necessary to consider the types of curves that one would expect from various types of cooperativity. In Figure 4 a standard saturation plot [velocity versus (S)], a double-reciprocal plot, and a Hill plot are shown for positive cooperativity, negative cooperativity, and classical Michaelis-Menten kinetics. When positive cooperativity is exhibited, the classi-

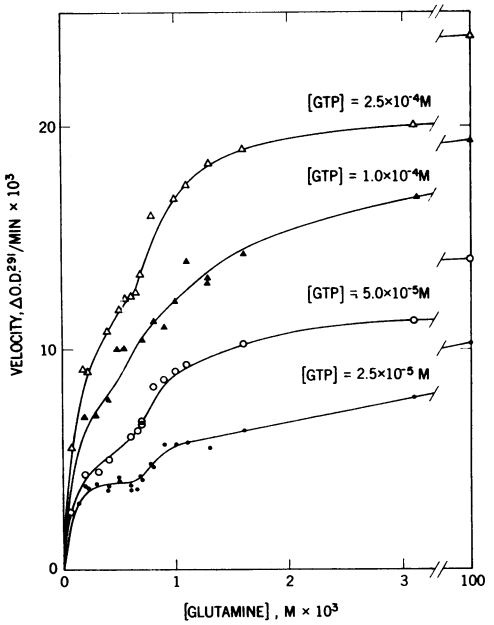

FIG. 1.—Dependence of reaction velocity of CTP synthetase on glutamine concentration at various GTP concentrations. The assay system (37°C) contained 0.02 M imidazole acetate, pH 7.2, 0.01 M Mg^{2+}, 7.5×10^{-4} M UTP (saturating), 7.5×10^{-4} M ATP (saturating), and 4 μg of pure CTP synthetase. Glutamine solutions were prepared immediately prior to assays.

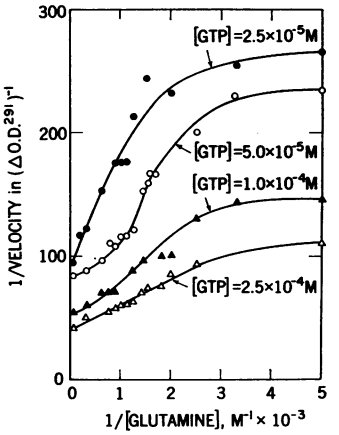

FIG. 2.—Double - reciprocal plots for data of Fig. 1. The data of Fig. 1 were replotted according to the Lineweaver-Burk equation.

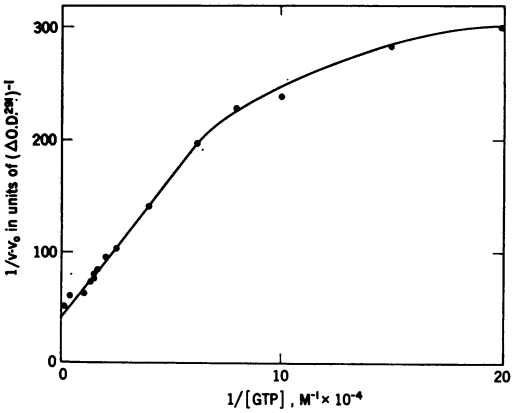

FIG. 3.—Double-reciprocal plot for the dependence of the CTP synthetase reaction velocity on GTP concentration. The assay conditions were the same as in Fig. 1, except that ($GluNH_2$) = 0.05 M and the GTP concentration varied as described in the figure.

cal sigmoid curve is obtained in the saturation plot, R_s is less than 81, the double-reciprocal plot is concave upward, and the Hill[9] coefficient is greater than 1 (in this particular example $n = 2$). For independent noninteracting sites the classical Michaelis-Menten curve is obtained, R_s equals 81, the double-reciprocal plot is linear, and the Hill coefficient is 1. For negative cooperativity the saturation plot looks qualitatively like a Michaelis-Menten curve but the R_s value is greater than 81, the double-reciprocal plot is concave downward, and the Hill coefficient is less than 1.

Although an R_s value of greater than 81 or a curve which is concave downward in a double-reciprocal plot is compatible with negative cooperativity, the absence of such an effect does not exclude the presence of this phenomenon. Since ligand-induced conformational changes may distort neighboring subunits,[2, 10]

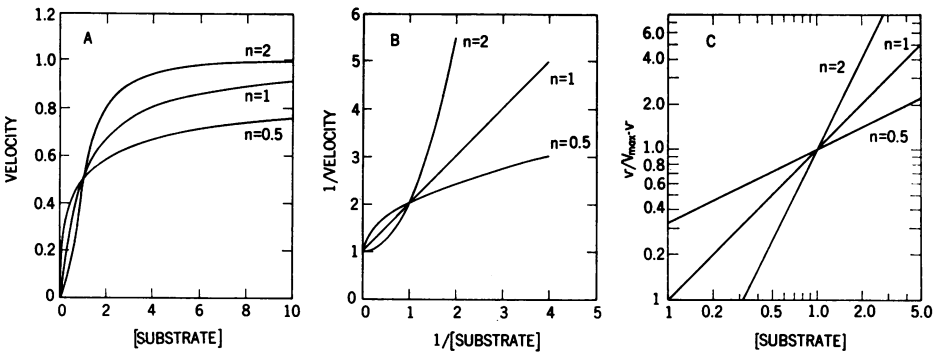

FIG. 4.—(*A*) Michaelis-Menten, (*B*) double-reciprocal, and (*C*) Hill plots of $v = V_m S^n/(K + S^n)$ for different n values. Theoretical curves drawn in cases in which $V_m = 1$, $K = 1$, and $n = 0.5$, 1, and 2.

it is not necessary that the active site be precisely the same for each of the molecular species ES, ES_2, ES_3, etc. Thus positive cooperativity in k_{cat} per site could exist at the same time as negative cooperativity with regard to binding. If the increase in catalytic power is sufficiently great, this will more than compensate for the decreased affinity of binding. An illustrative plot of v versus (S) is shown in Figure 5 together with the same data plotted in double-reciprocal and Hill-type plots. In such a case it is apparent that a plot of velocity versus substrate may give an R_s value less than 81 or equal to 81 even though negative

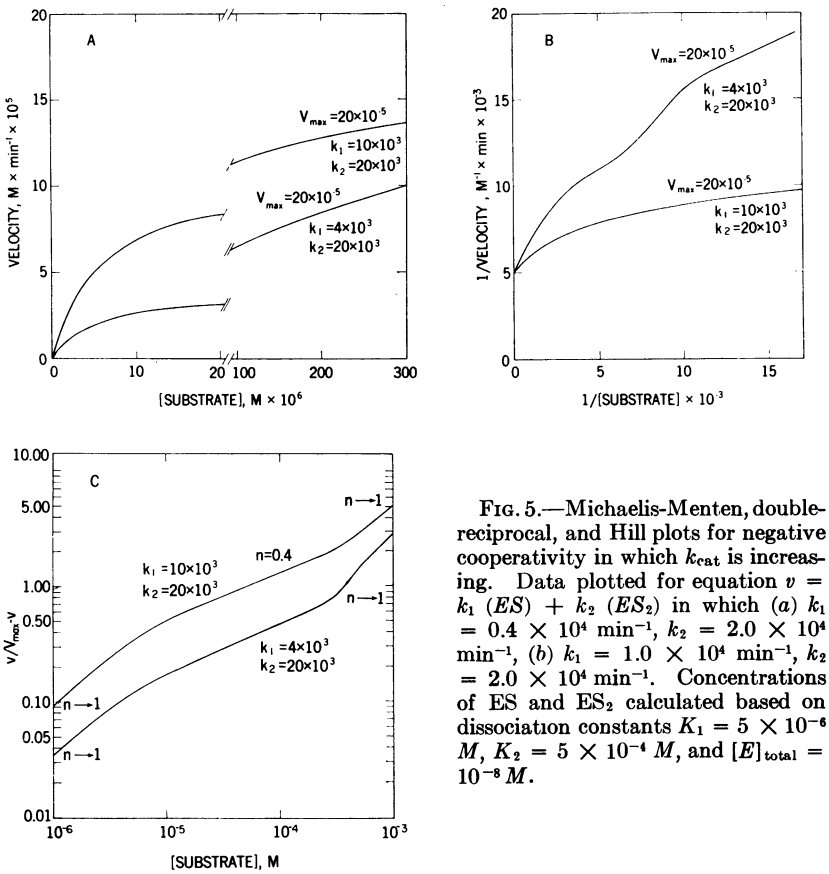

FIG. 5.—Michaelis-Menten, double-reciprocal, and Hill plots for negative cooperativity in which k_{cat} is increasing. Data plotted for equation $v = k_1 (ES) + k_2 (ES_2)$ in which (*a*) $k_1 = 0.4 \times 10^4$ min^{-1}, $k_2 = 2.0 \times 10^4$ min^{-1}, (*b*) $k_1 = 1.0 \times 10^4$ min^{-1}, $k_2 = 2.0 \times 10^4$ min^{-1}. Concentrations of ES and ES_2 calculated based on dissociation constants $K_1 = 5 \times 10^{-6}$ M, $K_2 = 5 \times 10^{-4}$ M, and $[E]_{total} = 10^{-8}$ M.

cooperativity with respect to binding was occurring. In these cases the R_s value by itself would not be diagnostic but the double-reciprocal plot or the Hill coefficient may be revealing, as deviations of the types shown in Figure 5 would be expected.

When these criteria are applied to the CTP synthetase data, many of the characteristics of negative cooperativity are seen to be present—i.e., high R_s values, and deviations on the double-reciprocal plot—but the curve is significantly different. A distinct leveling occurs in the plot of v versus (S) which is reminiscent of a similar leveling in the plot of v versus log (S) calculated previ-

ously[3] for a case of negative cooperativity. When similar calculations are made for a tetrameric protein, it appears that such a leveling can occur if both negative and positive cooperativity are present for the same ligand. For example, if the binding of the first glutamine molecule makes it easier for the second to bind, which in turn makes it more difficult for the third glutamine molecule, and this in turn increases the affinity of the fourth glutamine, such leveling will occur. A combination of negative and positive cooperativity in k_{cat} and negative cooperativity plus Michaelis-Menten binding can also lead to leveling in the plot of v versus (S). The typical downward deviation in the double-reciprocal plot indicating negative cooperativity is observed for the effector GTP.

Discussion.—The molecular events which could explain negative homotropic kinetics are (1) ligand-induced conformational changes which affect subunit interactions, (2) electrostatic repulsion between ligands, (3) nonidentical peptide chains having active sites with different binding constants as in isozymes, (4) two or more polymorphic forms of the same enzymes, (5) geometric arrangements of identical chains which produce nonidentical sites either because of static geometry or because of subunit conformational changes during the association of subunits, and (6) a combination of two or more of these alternatives. Electrostatic effects would seem to be excluded for the uncharged glutamine and unlikely to produce such large changes in the case of GTP for the reasons cited earlier.[2] The individual subunits of the CTP synthetase tetramer appear to be identical[7] but slight differences in amino acid sequence or association to produce different sites as suggested in alternatives (3), (4), and (5) may make some contribution to the unusual pattern. However, it is readily shown that the leveling in the plot of v versus (S) observed for glutamine or GTP can only be obtained if at least two of the sites exhibit ligand-induced negative cooperativity. Thus, alternative (1) is required to explain the kinetic pattern of CTP synthetase. Ligand-induced changes in the subunit interactions, furthermore, would explain the influence of GTP on the glutamine curves.

The sequential model for subunit interactions[2, 3] is based (1) on the induced-fit assumption that the conformational change induced in a flexible protein depends on the structure of the ligand and (2) on the assumption that the nature and amount of the distortion which is transmitted from one subunit to neighboring subunits will depend on the nature and strength of the subunit interactions. In one simple illustrative example it was shown[3] that variation of one parameter for subunit interactions, K_{BB}, could give rise to positive cooperativity (when $K_{BB} > 1$), negative cooperativity (when $K_{BB} < 1$), and Michaelis-Menten kinetics (when $K_{BB} = 1$). Such a model is ideally suited for the complex phenomena observed for CTP synthetase. No two-state[12] or even three-state or four-state model could explain the interlocking combinations of negative and positive cooperativity. This behavior, however, is readily explainable if it is assumed that the conformational changes induced by GTP are not necessarily identical to those induced by UTP or glutamine or ATP. If the conformational changes are not identical, the nature and strength of the subunit interactions need not be identical. In that case it is not surprising that one ligand can produce positive cooperativity and another ligand negative cooperativity.

Moreover, the effect of an added ligand will depend on the intermediate conformational state of the protein and hence on the ligands already attached to it. The alternation from negative to positive cooperativity in the sequential binding of a single ligand, for example, glutamine, and the change in the glutamine effects caused by the binding of the effector, GTP, are readily understandable on this basis.

Using the criteria developed in Figures 4 and 5, we examined the published literature and found numerous examples in which kinetic data could be explained by negative cooperativity. A few illustrative examples follow. Okazaki and Kornberg,[13] studying deoxythimidine kinase, recognized that their saturation plots, although superficially similar to Michaelis-Menten plots, were not correct quantitatively and that two separate K_m values for deoxythimidine would be needed to fit the data. Homoserine dehydrogenase,[14] mitochondrial isocitric dehydrogenase,[15] human heart lactic dehydrogenase,[16] and glutamic dehydrogenases from various sources[17–20] also show the characteristic deviations of negative cooperativity when the data are plotted in double-reciprocal plots like those of Figures 4 and 5. The phosphoenolpyruvate carboxylase of Corwin and Fanning[1] shows the same type of leveling in a plot of v versus (S) as was found for CTP synthetase. A particularly interesting example is aspartyl transcarbamylase,[21] the CTP and GTP homotropic data for which are plotted in Figure 6. It is seen that both of the Hill coefficients are less than 1, indicating negative cooperative effects. In this case negative cooperativity could explain a phenomenon which has remained puzzling, i.e., the failure to observe complete inhibition with GTP and CTP.[21] From the negative cooperativity plots it can be calculated that the R_s value is approximately 1000 and therefore nearly 0.1 M CTP would be required to obtain complete inhibition. This is a far higher concentration than would be calculated from Michaelis-Menten kinetics and hence far higher than the concentrations actually used in the experiments. Negative cooperativity can explain the apparent discrepancy between four binding sites for CTP and succinate[22] in a protein with six catalytic and six regulatory subunit.[23, 24] Finally, data[25, 26] on binding to immunoglobulin M antibodies show the deviations characteristic of negative cooperativity.

Although detailed studies will be needed to eliminate alternatives (2) through

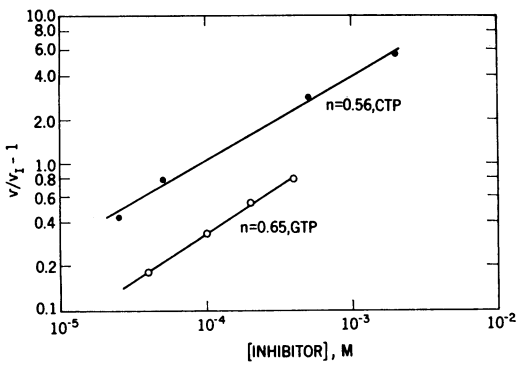

FIG. 6.—Hill plot for CTP and GTP inhibition of aspartyl transcarbamylase. Data taken from Gerhart and Pardee[21] and plotted according to the equation: Log $(v/v_i - 1)$ = log $1/K_i$ + n log (CTP), where v is activity in the absence of inhibitor, and v_i is activity in the presence of inhibitor.

(6) in each of these cases and in others which might be cited, evidence already exists for many of the enzymes which makes those alternatives unlikely. Thus, in certain cases identity of peptide chains has been demonstrated that has eliminated the alternatives of isozymes or pleomorphic forms. In other cases the enzyme contains both positive homotropic effects for some effectors and negative homotropic effects for others. Thus, it seems that the best working hypothesis at the moment would be the assumption that most of the kinetic evidence which suggests negative cooperative behavior is the result of the ligand-induced conformational changes suggested in alternative (1).

As a consequence of these studies, tentative answers to our questions can be given. First, negative cooperativity is apparently not a feature of a few odd enzymes but is rather a pervasive pattern in many. The fact that many of the data mentioned above were collected in unrelated studies indicates that many more cases will come to light when deliberate attempts are initiated to uncover such phenomena. Second, the procedures discussed here can be used as diagnostic approaches to negative cooperativity phenomena. As discussed in relation to Figures 4 and 5, a Hill coefficient less than 1, a biphasic double-reciprocal plot, and an R_s value greater than 81 are strong indications of negative cooperativity in a particular enzyme. The absence of one of these effects, however, does not exclude the existence of this phenomenon. Third, there is evidence that negative cooperativity is not identified with a special type of enzyme. Glyceraldehyde-3-phosphate dehydrogenase is an enzyme which does not have a regulatory site. CTP synthetase, deoxythimidine kinase,[13] homoserine dehydrogenase,[14] isocitrate dehydrogenase,[15] aspartyl transcarbamylase,[21] phosphoenolpyruvate carboxylase,[1] and glutamic dehydrogenases[17–20] appear to have regulatory sites. In all these cases negative cooperativity is indicated for at least one effector. In many of these cases positive cooperativity with other effectors is also observed. The wide range and complexity of these relationships lead one to conclude that conformational changes which depend on the specific ligand inducing the changes represent a simple unifying phenomenon which explains the diversity of individual patterns. It also explains the accompanying kinetic behavior which is so important in metabolic control.

The authors acknowledge the invaluable technical assistance of Miss Mary Gilchrist and Miss Phyllis Koshland.

* This work was supported in part by research grants from the National Institutes of Health (AM 09765) and the American Cancer Society (IN-87B).

† On leave of absence from the Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel.

[1] Corwin, L. M., and G. R. Fanning, *J. Biol. Chem.*, **243**, 3517 (1968).

[2] Conway, A., and D. E. Koshland, Jr., *Biochemistry*, **7**, 4011 (1968).

[3] Koshland, D. E., Jr., G. Nemethy, and D. Filmer, *Biochemistry*, **5**, 365 (1966); Kirtley, M. E., and D. E. Koshland, Jr., *J. Biol. Chem.*, **242**, 4192 (1967).

[4] Long, C. W., and A. B. Pardee, *J. Biol. Chem.*, **242**, 4715 (1967).

[5] Lieberman, I., *J. Am. Chem. Soc.*, **77**, 2661 (1955).

[6] Lieberman, I., *J. Biol. Chem.*, **222**, 765 (1956).

[7] Long, C. W., A. Levitzki, L. Houston, and D. E. Koshland, Jr., *Federation Proc.*, **28**, 342 (1969).

[8] Tris and Cl^- inhibit the enzyme.[4]

[9] Hill, A. J., *Biochem. J.*, **7**, 471 (1913).

[10] Haber, J. E., and D. E. Koshland, Jr., these PROCEEDINGS, **58**, 2087 (1967).
[11] Monod, J., J. Wyman, and J.-P. Changeux, *J. Mol. Biol.*, **12**, 88 (1965).
[12] The symmetry or concerted model of Monod, Wyman, and Changeux[11] predicts only positive homotropic effects.
[13] Okazaki, R., and A. Kornberg, *J. Biol. Chem.*, **239**, 275 (1964).
[14] Datta, P., and H. Gest, *J. Biol. Chem.*, **240**, 3023 (1965).
[15] Bernofsky, C., and M. F. Utter, *Biochim. Biophys. Acta*, **132**, 244 (1967).
[16] Niesselbaum, J. S., and O. Bodansky, *J. Biol. Chem.*, **236**, 323 (1961).
[17] LéJohn, H. B., and S. Jackson, *J. Biol. Chem.*, **243**, 3447 (1968).
[18] Corman, L., and N. O. Kaplan, *Biochemistry*, **4**, 2175 (1965).
[19] Corman, L., and N. O. Kaplan, *J. Biol. Chem.*, **242**, 2840 (1967).
[20] Wiggert, B. O., and P. O. Cohen, *J. Biol. Chem.*, **241**, 210 (1966).
[21] Gerhart, J. C., and A. B. Pardee, *Federation Proc.*, **23**, 727 (1964); *J. Biol. Chem.*, 237, 891 (1962).
[22] Changeux, J.-P., J. C. Gerhart, and H. K. Schachman, *Biochemistry*, **7**, 531 (1968).
[23] Weber, K., *Nature*, **218**, 1116 (1968).
[24] Wiley, D. C., and W. N. Lipscomb, *Nature*, **218**, 119 (1968).
[25] Onoue, K., Y. Yagi, A. L. Grossberg, and D. Pressman, *Immunochemistry*, **2**, 401 (1965).
[26] Onoue, K., A. L. Grossberg, Y. Yagi, and D. Pressman, *Science*, **162**, 574 (1968).